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Characterisation of the membrane-extrinsic domain of the TatB component of the twin arginine protein translocase

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ABSTRACT

The twin arginine protein transport (Tat) system transports folded proteins across cytoplasmic membranes of bacteria and thylakoid membranes of plants, and in *Escherichia coli* it comprises TatA, TatB and TatC components. In this study we show that the membrane extrinsic domain of TatB forms parallel contacts with at least one other TatB protein. Truncation of the C-terminal two thirds of TatB still allows complex formation with TatC, although protein transport is severely compromised. We were unable to isolate transport-inactive single codon substitution mutations in *tatB* suggesting that the precise amino acid sequence of TatB is not critical to its function.

Structured summary:
TatA physically interacts with **TatA** by two hybrid (View interaction)
TatB and **TatC** bind by molecular sieving (View interaction)
TatB physically interacts with **TatB** by two hybrid (View Interaction 1, 2)

1. Introduction

Protein transport across the bacterial cytoplasmic membrane is achieved by one of two general pathways. The Sec (general *sec*retory) pathway exports proteins in an unfolded conformation [1]. By contrast the *t*win *a*rginine protein *t*ransport (Tat) pathway transports folded proteins across the membrane, driven solely by the protonmotive force (Δp) [2,3]. Proteins are targeted to the Tat pathway by N-terminal signal peptides that harbour a conserved twin arginine motif [4].

The Tat system is found in most bacteria, in some archaea and in the thylakoid membranes of plant plastids. In Gram negative bacteria the Tat machinery is made up of three inner membrane proteins, TatA, TatB and TatC [5–8]. The TatA protein probably makes up the protein-translocating element of the Tat system. It forms homo-multimeric complexes of varying sizes which by electron microscopy analysis form channel-like complexes of differing diameter [9,10]. The TatB and TatC proteins form a separate complex that acts as the receptor for the Tat system. It contains TatB and TatC in a 1:1 ratio, with each protein present in multiple copies (e.g. [11–13]). Substrate proteins interact with the TatBC complex through their twin-arginine signal peptides [12–16]. Evidence suggests that TatA is subsequently recruited to the substrate-bound TatBC complex in a process that requires Δp [14,16].

The precise functions of the individual subunits within the TatBC complex are not fully resolved. Crosslinking experiments have implicated both TatB and TatC in signal peptide binding, with TatC probably recognising the twin arginine motif [16,17]. TatB shares sequence identity with TatA and it is likely that the proteins arose from a common ancestor [18]. Despite this structural similarity the two proteins have distinct functions in Tat transport and cannot substitute for one another [5]. Although *Escherichia coli* TatB is absolutely required for transport of physiological Tat substrates, some sensitive reporter proteins can be exported at very low levels in a *tatB* mutant strain, suggesting that TatB may not be mechanistically essential for operation of the Tat pathway [19,20]. This TatB-independent transport can be enhanced by point mutations in the N-terminal six amino acids of TatA [20]. These findings are consistent with the observation that minimal Tat systems in some prokaryotes do not require TatB proteins [21,22].

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In this study we have further explored the role of TatB in *E. coli* Tat protein transport.

2. Materials and methods

2.1. Strains, plasmids, media and culture conditions

The following strains were used in this study: BTH101 (F^-, *cya-99*, *araD139*, *galE15*, *galK16*, *rpsL1* (Str^R), *hsdR2*, *mcrA1*, *mcrB1* [23]); SU202 (*lexA71*::Tn5, *sulA211*, *sulA* (op408/op+)::*lacZ* Δ(*lacIPOZYA*) 169/F′*lacI*^{q}*lacZ*Δ M15::Tn9 [24]); MC4100 (F^-Δ*lacU169 araD139 rpsL150 relA1 ptsF rbs flbB5301* [25]; DADE (As MC4100, Δ*tatABCD*, Δ*tatE* [26]); DADE-P (As DADE, *pcnB1 zad*-981::Tn10d [27]). Plasmids used in this work are listed in Table S1; construction of all of the plasmids and mutant libraries used in this study are described in Supplementary information. Strains were cultured aerobically at 37 °C in Luria–Bertani (LB) medium unless stated. The ability of strains to use TMAO as sole electron acceptor, or to grow in the presence of SDS were scored as described [27]. For TMAO reductase activity assays, strains were cultured anaerobically overnight in LB medium supplemented with 0.5% glycerol and 0.4% TMAO. Antibiotics were used at the following final concentrations: ampicillin (125 μg/ml), kanamycin (25 μg/ml) chloramphenicol (20 μg/ml for selection of plasmids based on pT25, and 100 μg/ml when screening for alleles of *tatB* that reduced export of the TorA–CAT fusion).

2.2. Protein methods

For the isolation of TatBC complexes, the protocol of Orriss et al. [28] was used with the exception that size exclusion chromatography was performed using a Superose-6 column. Protein standards thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), albumin (66 kDa) and carbonic anhydrase (29 kDa) (all purchased from Sigma) were used to calibrate the size exclusion column, with blue dextran (Sigma) used to calculate the void volume. SDS PAGE using the Tris-glycine and Tris-tricine buffer systems and immunoblotting were performed as described [29–31]. Rabbit polyclonal anti-HA antisera were purchased from Sigma and HRP-conjugated anti penta-his antiserum from Qiagen. TMAO reductase and β-galactosidase assays were performed as described [32–34].

3. Results

3.1. The membrane extrinsic domain of TatB self interacts

The predicted secondary structure organisation of *E. coli* TatB is shown in Fig. 1A. The N-terminal region shows homology to TatA.

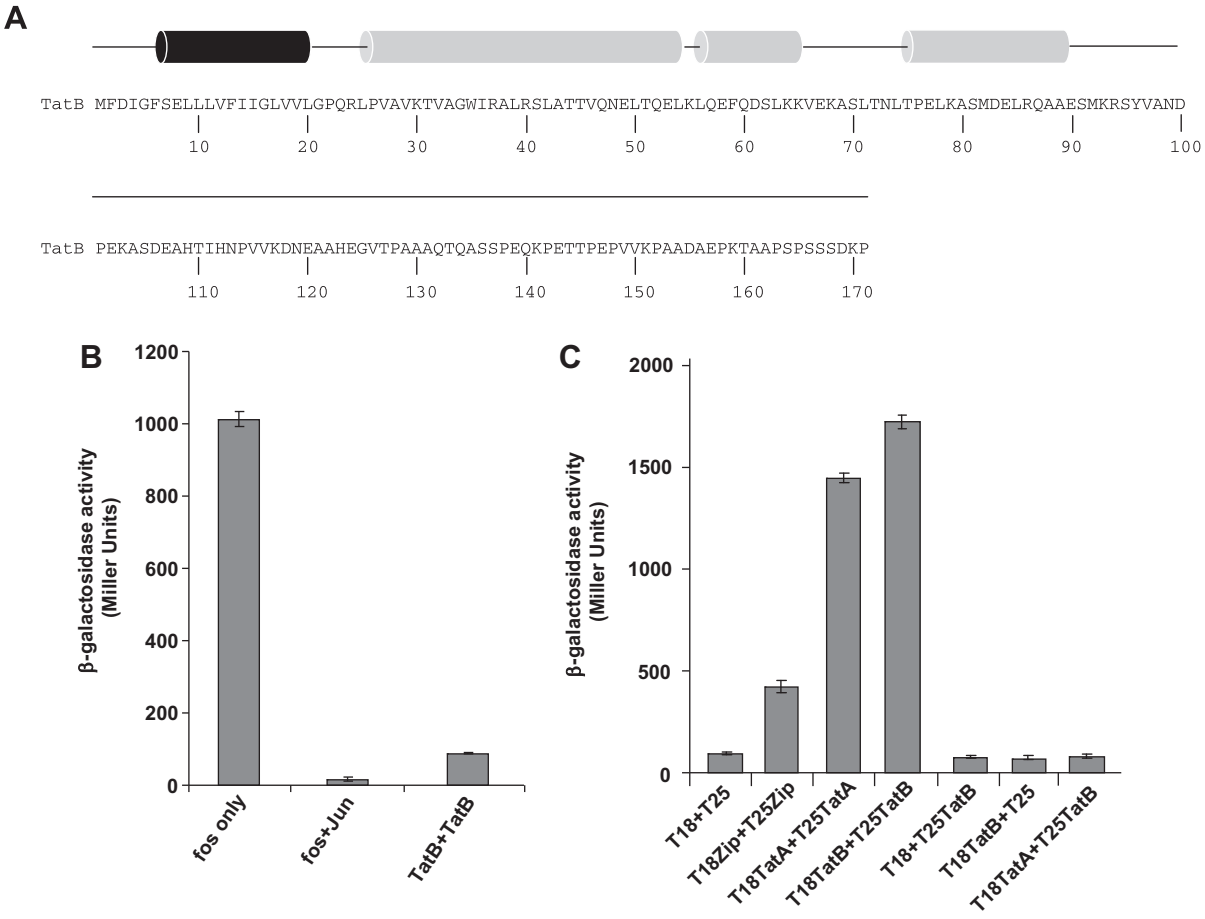

Fig. 1. Dimerisation of the membrane extrinsic domain of TatB. (A) The amino acid sequence of *E. coli* TatB is shown below the predicted secondary structure for the protein (determined using JPred [44]). Predicted α-helical regions are shown as cylinders, with the transmembrane helix in black. (B) Repression of *lacZ* expression in strain SU202 by heterodimeric LexA fusion proteins formed through interaction of fos and jun zippers (produced from plasmids pMS604 and pDP804, respectively) or through dimerisation of the membrane-extrinsic domain of TatB (produced from plasmids pMSB2 and pDPB1). The column marked fos is strain SU202 harbouring a single plasmid (pMS604) and serves as a negative control. (C) Self-interactions between the membrane extrinsic domains of TatA (amino acids 22–89) or TatB (amino acids 22–171) assessed using the adenylate cyclase bacterial two hybrid system. T18Zip and T25Zip are plasmids producing the leucine zipper region of GCN4 fused to the two separate fragments of adenylate cyclase and serve as a positive control. Error bars represent the standard error of the mean ($n = 3$).

This region comprises an N-terminal transmembrane helix (residues 7–20) followed by a Gly-Pro hinge dipeptide (with the Gly being conserved in TatA) and then a basic amphipathic helix (residues 26–40). The central region of the protein from residues 41–91 is predicted to be predominantly helical. Within this central region residues 41–54 are well-conserved in the γ-Proteobacteria and form an amphipathic helix that may be a continuation of the preceding amphipathic helix, and residues 56–65 and 75–89 are also proposed to form helices. The C-terminal region of the protein from residue 90 onwards is predicted to have a predominantly random coil conformation. It is plausible that the amphipathic helical regions of TatB interact with the membrane. Indeed, protein engineering studies show that the portion of TatB following the transmembrane helix (the membrane-extrinsic domain) has peripheral interactions with the membrane [35].

Crosslinking studies have shown that the TatB protein is in intimate contact with up to three other TatB proteins within the TatBC complex [33–35]. While TatB self-interactions appear to involve the transmembrane helix, disulphide and amine-specific crosslinks have also been detected between membrane-extrinsic domains [34,35]. It is unclear whether these crosslinks reflect specific protein–protein interactions between the membrane-extrinsic domains or merely indicate that the domains are in close proximity because they are attached to interacting transmembrane helices. To address this issue we have investigated the possible self-interactions of the TatB membrane-extrinsic domain using two in vivo approaches.

The LexA-based genetic system relies upon fusing proteins of interest to the C-termini of two LexA proteins that differ in the DNA operator sequences that they recognise [24]. *E. coli* reporter strain SU202 contains a hybrid operator sequence upstream of *lacZ* that is only bound when heterodimeric LexAs are formed through interaction of the fusion proteins. A control experiment in which two domains (fos and jun zippers) that are known to interact were fused to the two LexA proteins demonstrates the expected repression of *lacZ* expression (Fig. 1B). By contrast, when only one of the LexA proteins (in this case fused to the fos zipper sequence) is produced, high levels of β-galactosidase activity are detected, consistent with little or no repression of the *lacZ* promoter. When the membrane extrinsic domain of TatB is fused to the two LexA variants strong repression of *lacZ* expression is also observed. These observations indicate that in this protein interaction system TatB dimerisation is clearly detected.

To confirm these observations a bacterial two hybrid system was used, based around the reconstitution of adenylate cyclase activity, from two non-interacting fragments of the *Bordetella pertussis* adenylate cyclase [36]. Positive interactions are indicated by a high level of β-galactosidase enzyme activity through de-repression of the *lacZ* gene. Fig. 1C demonstrates that when the membrane-extrinsic domain of TatB is fused to the two fragments of

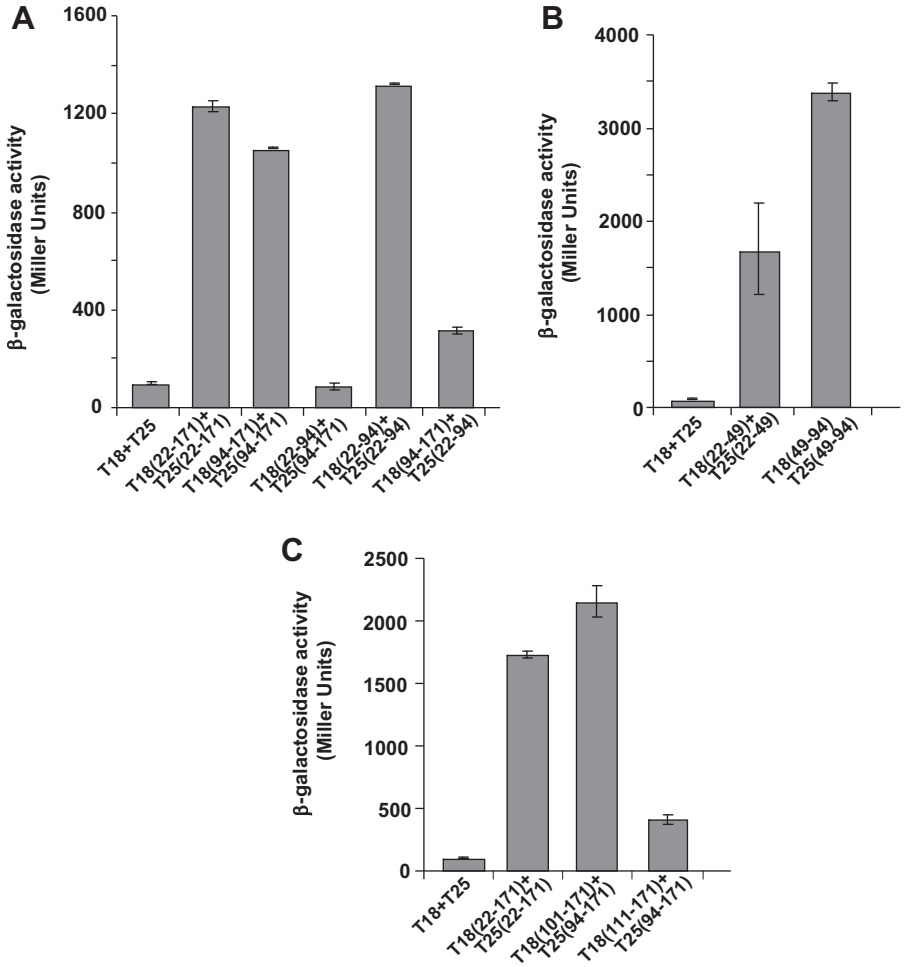

Fig. 2. TatB dimerises along its length in a linear manner. Fragments of *E. coli* TatB (amino acid positions shown in brackets) fused to either the N-terminal end of T18 or the C-terminal end of the T25 fragment of *B. pertussis* adenylate cyclase were assessed for their ability to interact in the bacterial two hybrid system. Error bars represent the standard error of the mean ($n = 3$).

adenylate cyclase a very high level of β-galactosidase was measured, and which was considerably higher than that seen for the positive control (the self-interaction between the leucine zipper region of the yeast protein GCN4 – [36]). When one of the *tatB*-containing plasmids was replaced by a vector without a *tatB* insert no interaction was detected, indicating that the interaction was between the TatB fragments present on the fusion proteins.

TatA and TatB are related proteins and they show a similar predicted domain organisation in their first 40 amino acids. However, no interaction between the membrane-extrinsic domains of TatA (amino acids 22–89) and TatB could be observed. Intriguingly a test for self–self interaction in the TatA membrane-extrinsic domain gave levels of β-galactosidase close to that seen for the TatB self-interaction. Interactions between the membrane-extrinsic portions of TatA molecules were unanticipated because the purified domain is a monomer in aqueous solution [37] which may point to a role for membrane interactions [37] in driving domain oligomerization. The observation of contacts between TatA membrane-extrinsic domains would be consistent with the disulfide crosslinking that can be observed between amphipathic helices for TatA in membranes [38].

3.2. The membrane-extrinsic domain of TatB dimerises in a parallel fashion and self-interaction primarily involves the predicted helical domain

In order to determine the regions of TatB required for homodimerisation, we designed two hybrid constructs that produce smaller domains of TatB. As shown in Fig. 2A, the region of TatB extending from amino acids 22–94 was able to homodimerise, as was the region from amino acids 94–171. However, the region from 22–94 did not show interaction with the region from 94–171, indicating that TatB interacts in a linear fashion along its length. We next divided the region from amino acids 22–94 into two parts – amino acids 22–49 and amino acids 49–94 and tested each of these for homodimerisation in the two hybrid system. Again both of these regions showed strong self interaction (Fig. 2B).

To further delimit the region of TatB involved in self-interaction, we used the clone containing DNA encoding amino acids 94–171 of TatB and truncated this by removal of DNA coding for the amino terminal 7 residues or the amino terminal 17 residues, to give constructs producing TatB amino acids 101–171 or 111–171 fused to the T18 fragment of adenylate cyclase. As shown in Fig. 2C, the construct producing amino acids 101–171 of TatB still displayed strong interaction with the 94–171 fragment of TatB fused to T25. However removal of an additional 10 amino acids gave a fusion protein that showed almost no interaction with the 94–171 fragment of TatB fused to T25. This could reflect a loss of self interaction in this region of the protein, or alternatively result from instability of the T18–$TatB_{111-171}$ fusion protein. None-the-less, taken together these results indicate that the self-interacting region of the membrane-extrinsic domain extends covers at least amino acids 22–100 of the protein.

3.3. The membrane extrinsic part of TatB is not required for TatBC complex formation

Previous studies have shown that truncation from the C-terminus of TatB to form a protein corresponding to the first 71 amino acids of TatB reduced, but did not abolish, Tat transport [39].

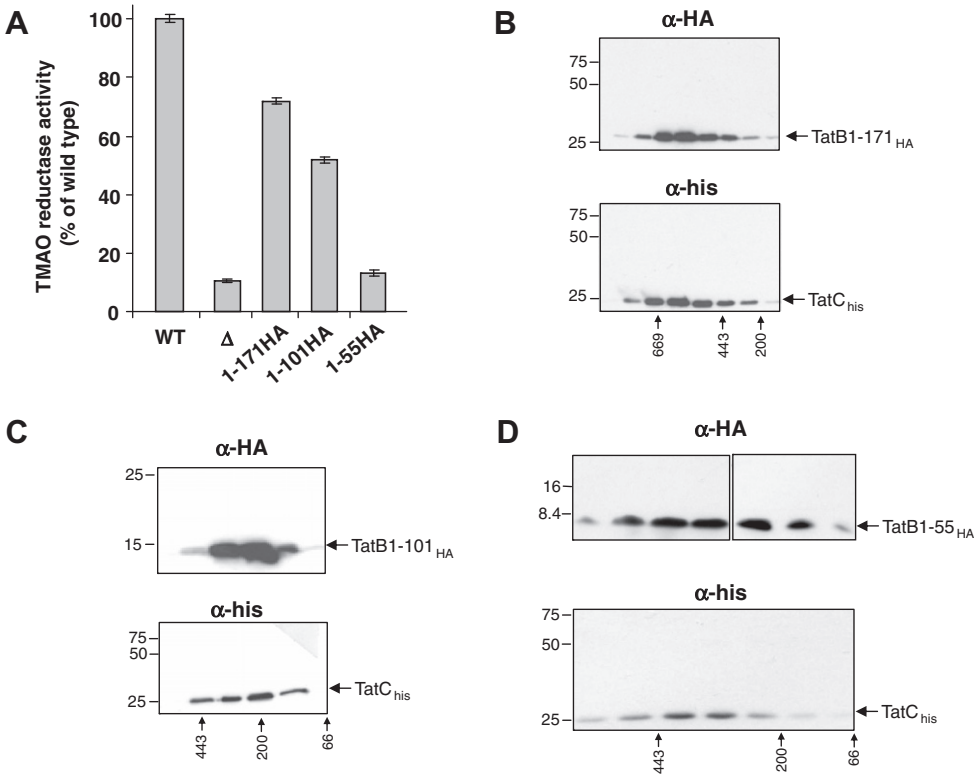

Fig. 3. The membrane extrinsic domain of TatB is not required for TatBC complex formation. (A) *E. coli* strain DADE-P harbouring pFAT405 (*tatA*$^+$) alone (Δ) or with a separate plasmid producing his-tagged TatC and either native TatB (WT) or a HA-tagged variant of TatB that was either full length (1–171HA), lacking the C-terminal 70 amino acids (1–101HA) or lacking the C-terminal 116 amino acids (1–55HA) was cultured anaerobically in the presence of TMAO, periplasmic fractions prepared and assayed for TMAO reductase activity. 100% activity was that determined for DADE-P producing plasmid encoded *tatA*, native *tatB* and $tatC_{his}$ (labelled WT in the figure). Error bars represent the standard error of the mean ($n = 3$). (B–D) Western blot analysis of proteins eluting from a Superose 6 size exclusion chromatography column. Samples were probed for the presence of TatC (using anti his-tag antisera) and TatB (using anti HA antisera). The sizes of molecular weight markers (kDa) are indicated to the left of each panel and the elution positions of protein standards (kDa) from the superose 6 column are indicated below the panels.

However more severe truncations gave barely detectable transport activities, indicating an important mechanistic function for the predicted amphipathic helix-containing region involved in self–self interactions [39]. One possibility for the loss of activity of the truncated TatB proteins is that the complex formed with TatC is disrupted. To investigate whether the membrane-extrinsic region of TatB is essential for formation or stability of the TatBC complex, we co-produced his-tagged TatC with truncated variants of TatB, and purified TatC-containing complexes from *E. coli* membranes. Two constructs were designed – one which produces a TatB protein comprising amino acids 1–55 of the protein (which approximates to a protein comprising the N-terminal transmembrane and adjacent amphipathic helices) and a second comprising amino acids 1–101 (which from the truncation analysis above delimits the self-interacting region of the protein). Since the truncated variants of TatB lacked the epitope binding site recognised by our TatB antiserum, it was also necessary to introduce a hemagglutinin (HA) epitope tag at the C-terminus of the TatB proteins to facilitate their detection by immunoblotting.

As shown in Fig. 3A, when a plasmid producing full length TatB with a C-terminal HA tag and his-tagged TatC was co-transformed with a *tatA*-encoding plasmid in a strain devoid of chromosomally-encoded *tat* genes, active Tat systems were formed, as evidenced by the high level of activity of the Tat-targeted enzyme trimethylamine-*N*-oxide (TMAO) reductase in the periplasmic fraction. A HA-tagged TatB protein comprising the first 101 amino acids of the protein was also competent to support translocation of TMAO reductase to the periplasm (Fig. 3A). By contrast, a fragment of TatB covering only the first 55 amino acids of the protein supported only a very low level of protein transport that was indistinguishable from the negative control. However, phenotypic growth tests showed that this form of TatB allowed growth on minimal medium with TMAO as sole electron acceptor and on agar plates containing 2% SDS (both of which require an active Tat system; [27]) indicating that this TatB truncation has residual Tat function. These phenotypic observations are broadly consistent with a previously reported truncation analysis [39].

To examine complex formation between TatB and TatC, the proteins were overproduced in the absence of TatA and in a strain devoid of chromosomally-encoded Tat components. Proteins were extracted from the membrane fraction by treatment with digitonin, TatC-containing complexes were isolated by nickel affinity chromatography and identified by western blotting using an anti-histag antibody. TatC-containing fractions were concentrated and subjected to a further round of purification by size exclusion chromatography. Fig. 3B shows that when co-produced with full length TatB (carrying a C-terminal HA tag), his-tagged TatC elutes from the gel filtration column in fractions corresponding to a molecular mass between 443 and 669 kDa (lower panel). The same column fractions also contained TatB protein, revealed by western blot analysis with anti-HA antiserum (upper panel). The size of the purified complex is similar to that observed for the TatBC complex containing non-tagged TatB [28].

It can be seen from Fig. 3C and D that C-terminal truncation of TatB to either 101 or 55 amino acids in length did not affect the ability of the protein to form a complex with TatC. In both cases TatB was detected with the anti-HA antiserum co-eluting in the same fractions from the gel filtration column as his-tagged TatC. In each case the TatBC complex formed from truncated TatB proteins appeared smaller on size exclusion chromatography than the complex formed with full length TatB protein, consistent with the removal of a significant proportion of the total mass of TatB. As expected, the truncated TatB proteins also migrated faster on SDS PAGE. Taken together these results show that the C-terminal two thirds of TatB is not important for the formation or stability of the TatBC complex.

3.4. Random mutagenesis suggests that TatB is resistant to inactivation by amino acid substitution

To further explore the function of TatB, we developed a screen to isolate alleles of *tatB* that were unable to support Tat-dependent protein transport. The first part of the screen utilised a previously reported positive selection for loss of Tat function [40]. A TorA signal peptide is able to direct Tat-dependent targeting of the enzyme chloramphenicol acetyltransferase (CAT) to the periplasm. Since only cytoplasmically-located CAT can detoxify chloramphenicol, cells with a fully functional Tat system and producing the TorA signal peptide-CAT fusion protein from plasmid pTTC1 are sensitive to chloramphenicol concentrations of 100 μg/ml. By contrast, cells with a partial or complete defect in Tat-dependent export can grow on media containing this level of chloramphenicol. As this screen can not readily distinguish between mutations that reduce Tat activity and those that completely inactivate the pathway, a second part of the screen took advantage of the fact that full inactivation of the Tat pathway renders *E. coli* K12 unable to grow in the presence of 2% SDS [27]. Only *tatB* alleles that supported growth on chloramphenicol but did not allow growth on SDS were considered to be inactive.

Two *tatB* mutant libraries were constructed, one which had a low error frequency (0.25% error rate; approximately 1–2 mutations per gene) and 600,000 individual members, and a second that had a higher error frequency (1.0%; around 5 mutations per gene) and 900,000 members. The plasmid libraries (which harbour a wild type copy of *tatA* and *tatC* along with mutagenised *tatB*) were transformed into *E. coli* strain DADE (which lacks chromosomal *tat* genes) containing plasmid pTTC1. After transformation of this strain with 40,000 clones from the low error rate library, some 10% or so of the transformants were able to grow on chloramphenicol-containing media. 134 individual colonies were selected at random and replica spotted onto LB medium containing 100 μg/ml chloramphenicol or 2% SDS. 65 of these showed good growth on chloramphenicol but failed to grow on SDS-containing media, and the *tatB* genes were amplified by colony PCR and sequenced to indentify the nature of the inactivating mutation(s). All of these (listed in Table S2) contained either a premature stop codon or a frame shift mutation within the first 60 codons of *tatB* (i.e. the essential region of TatB identified by truncation analysis [39]) or affected the translation of *tatC* or *tatA* (by giving rise to a TatA–TatB fusion). Given that such mutations were present at low frequency in the original library (one frame-shift and two stop codon mutations from 20 clones sequenced) but were massively amplified during the screening process, this indicates that there are likely to be relatively few single amino acid substitutions that inactivate TatB.

We also undertook screening of the higher error rate library. From 22 clones that conferred resistance of strain DADE/pTTC1 to 100 μg/ml chloramphenicol and sensitivity to 2% SDS, the majority again harboured multiple codon substitutions coupled with stop codons or frame shifts (Table S2). The three clones that did not contain such mutations in this region of *tatB* harboured multiple amino acid changes in both the transmembrane and amphipathic helical portions of the protein. These findings are again consistent with the idea that TatB is relatively refractory to inactivation by mutagenesis.

4. Discussion

In this study we have focused on TatB protein:protein interactions, and in particular the involvement of the membrane-extrinsic region of the protein. Two hybrid analysis of the membrane extrinsic domain demonstrated that the self-interacting region of TatB extends to at least the first 100 amino acids of the protein.

Moreover we have shown that the protein interacts in a parallel fashion which indicates that the membrane-extrinsic region of TatB adopts an extended conformation. Such a linear arrangement of adjacent membrane-extrinsic domains would be consistent with the observation that TatB variants containing single cysteine substitutions in the early part of this region can be disulfide cross-linked [35]. Our two hybrid analysis demonstrates no specific interaction between the membrane-extrinsic domains of TatA and TatB, even though both membrane-extrinsic domains strongly homo-dimerise in this system. This is consistent with earlier chemical crosslinking studies using lysine-specific reagents [33,34] which failed to detect contacts between these two proteins.

Truncation analysis has shown that successive removal of blocks of 10 amino acids from the C-terminal end of TatB leads to a gradual loss of function [26]. However we found no apparent effect on TatBC complex formation or stability when the TatB component comprised only the first 55 or 101 amino acids, indicating that only the transmembrane and/or adjacent amphipathic helical region is required for complex formation with TatC. The reason for the loss of activity of C-terminally truncated forms of TatB is not clear, but it apparently does not correlate with the ability to bind to TatC.

Various site-directed mutagenesis studies have been undertaken on both TatA and TatB proteins in an attempt to find functional amino acid residues that define TatA and TatB family proteins. The amphipathic helix region of TatA is highly sensitive to inactivation by point mutation whereas substitutions in the same region of TatB had very little effect on TatB function [35,38,40–42]. In this study we took an unbiased approach to isolate point mutations that resulted in a complete loss of TatB function. Consistent with previous directed mutagenesis studies we did not isolate any fully inactivating single amino acid substitutions. Instead we enriched for stop codon or frame shift mutations in the essential coding region of TatB. Taken together this evidence strongly suggests that there are very few, if any amino acid side-chains that are essential for the function of TatB. This contrasts markedly with the numerous inactivating mutations that have been isolated in TatA and it underscores the different roles that these proteins play in Tat-dependent protein transport.

Previous evidence indicates that TatB forms part of the signal peptide binding site [16,17,43]. However, whilst TatC contacts signal peptides in the vicinity of the conserved twin arginine motif, TatB contacts signal peptides along the full length of the h-region [16,17]. The extent and sequence-independent nature of this interaction suggests that a single amino acid substitution in TatB is unlikely to completely eliminate signal peptide interactions with the TatBC complex. Other functions of TatB, such as binding to TatC or TatA might also be anticipated to also involve extensive protein–protein interactions that may not be abolished by single amino acid substitutions. TatA, by contrast, may need finer control of interactions with other proteins and membrane lipid because it needs to modulate these interactions at different points of the transport cycle.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.01.016.

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